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Prevalence of *Staphylococcus aureus* and of methicillin-resistant *S. aureus* (MRSA) along the production chain of dairy products in north-western Greece

Panagiotis Papadopoulos [a, *], Theofilos Papadopoulos [b], Apostolos S. Angelidis [c], Evridiki Boukouvala [b], Antonios Zdragas [b], Anna Papa [d], Christos Hadjichristodoulou [e], Daniel Sergelidis [a]

[a] Laboratory of Hygiene of Foods of Animal Origin, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[b] Greek Agricultural Organization—DIMITRA, Veterinary Research Institute of Thessaloniki, 57006 Thermi, Greece
[c] Laboratory of Milk Hygiene and Technology, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[d] Department of Microbiology, Medical School, Aristotle University of Thessaloniki, 54 124 Thessaloniki, Greece
[e] Department of Hygiene and Epidemiology, Medical School, University of Thessaly, 41 222 Larissa, Greece

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ABSTRACT

The objective of this study was to estimate the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in the production chain of dairy products. Of 367 tested samples (36 bulk tank milk (BTM), 19 dairy products, 72 human, 185 animal, 55 equipment), 212 (57.8%) were found positive for *S. aureus*. Almost all isolates (99.6%) were resistant to at least one antimicrobial and 13.3% were multi-drug resistant (MDR), exhibiting resistance to three or more antibiotic classes. Eleven samples (3%) were found contaminated by MRSA carrying the *mec*A gene. None of the MRSA isolates carried the *mec*C or the Pandon-Valentine leucocidin (PVL) genes. Four *spa* types were identified among the MRSA isolates: t127, t3586, t1773, t4038, with t127 being the most prevalent (7 out of 11). Two of them, t3586 and t1773, were isolated for the first time in Greece. Furthermore, Pulse-Field Gel Electrophoresis (PFGE) analysis indicated clonal circulation through the dairy production chain. The presence of MDR *S. aureus*, and especially MRSA, in animals and dairy products represents a potential threat for the spread of this pathogen in the community. The results indicated that human, animal and environmental sources could be involved in the contamination of dairy products along their production chain and therefore further investigation of contamination sources is needed to control the dispersion of MRSA in the community.
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1. Introduction

Staphylococcus aureus (*S. aureus*) is a versatile virulent opportunistic pathogen for humans and animals and is responsible for various infections, such as wound infections and toxin-mediated syndromes (food-poisoning, scalded skin syndrome and toxic-shock syndrome) as well as systemic and life-threatening diseases such as endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis and bacteremia (Chambers and DeLeo, 2009; Deurenberg and Stobberingh, 2008; Tauxe, 2002). *S. aureus* is capable of acquiring antibiotic resistance determinants and therefore *S. aureus* isolates often exhibit resistance to multiple classes of antimicrobial agents (Rybak and LaPlante, 2005). Methicillin-resistant *S. aureus* (MRSA) is practically resistant to all available β-lactam antimicrobial drugs. MRSA represents a serious public-health issue due to its ability to colonize and infect humans and animals (Petinaki and Spiliopoulou, 2012).

MRSA was first recognized as a hospital-associated pathogen (HA-MRSA) (Jevons et al., 1963). However, since 1990, community-associated MRSA (CA-MRSA) began to cause infections outside the health-care environment (Rybak and LaPlante, 2005). In recent years, the incidence of MRSA infections has increased in livestock and a third epidemiological type was recognized, the livestock-associated MRSA (LA-MRSA) (Graveland et al., 2011). While

* Corresponding author. Laboratory of Hygiene of Foods of Animal Origin, Department of Hygiene and Technology of Foods of Animal Origin, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece.
E-mail address: ppanagie@vet.auth.gr (P. Papadopoulos).

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humans are considered as the main reservoir for new pathogenic strains affecting livestock (Cuny et al., 2013), animals can act as a source of *S. aureus* zoonotic infections, especially the clones that possess no host specificity (Peton and Le Loir, 2014). It has also been demonstrated that handling and consumption of MRSA-contaminated food of animal origin could provide a potential vehicle for transmission to humans (EFSA, 2009; Feingold et al., 2012).

Very few studies have focused on the prevalence of MRSA along the production chain from lactating ruminants to ready-to-eat (RTE) dairy products. Such studies may help to identify whether isolates present on the living animal will end up in the dairy products or whether the isolates found in final products originate from contaminations along the food chain (Wendlandt et al., 2013). In the present study we estimated the prevalence of *S. aureus* and MRSA along the dairy production chain, from farm to RTE products. Pulse Field Gel Electrophoresis (PFGE), *spa* typing, as well as antimicrobial resistance typing were used to determine the relatedness among the MRSA isolates.

2. Materials and methods

2.1. Sample collection

A total of 367 samples were collected between January and May of 2016 from two dairy plants and the corresponding 24 dairy farms providing milk (bovine, ovine and caprine) to these plants in Epirus (north-western Greece). Different types of samples were collected: 36 bulk-tank milk (BTM) samples (10 bovine, 19 ovine, 5 caprine and 2 mixed ovine and caprine); 19 samples of dairy products (prepackaged soft and hard cheese, yogurt and butter from the dairy plants' storehouses); 257 nasal swabs (72 from humans, 35 from cows, 111 from sheep and 39 from goats) and 55 swabs from dairy plants (equipment surfaces).

For BTM sampling, the milk was stirred before sampling and 50 ml from storage tanks (of each farm and dairy plant) were aseptically collected into sterile tubes. Nasal samples from animals and humans were taken from both nares using sterile, cotton-tipped swabs. Swabs were also used for sampling the dairy plants' equipment surfaces (cheese cutters and pressers, knives, knobs, tanks). The swabs were immediately placed into 10 ml Tryptone Soy broth (TSB; LAB M Limited, Lancashire, United Kingdom) with 6.5% w/v NaCl (Merck KGaA, Darmstadt, Germany) and 0.3% yeast extract (LAB M).

All human samples were obtained voluntarily and all participants declared receiving no recent (within the preceding 3 months) antimicrobial treatment. The farm owners consented to animal sampling and also declared no recent (1 month) antimicrobial treatments to their animals. All collected samples were transported to the laboratory in refrigerated (*ca*. 2 °C) containers within 6 h after sampling and processed immediately.

2.2. Isolation and identification of S. aureus

Isolation and enumeration of staphylococci was carried out as previously described (Sergelidis et al., 2012). Enumeration was performed only in milk and dairy products. Ten-ml portions of milk and 10 g from dairy products were aseptically removed from each sample, placed into stomacher bags containing 90 ml TSB with 6.5% w/v NaCl, homogenized for 2 min (Lab Blender 400, A. J. Seward and Co. Ltd. London) and then 10-fold serial dilutions were prepared in the same broth. One ml from each dilution was pour-plated into Baird Parker agar (BPA; LAB M) supplemented with egg yolk tellurite (LAB M). For the detection of less than 10 CFU (per g or ml of sample), 1 ml of milk was plated onto BPA, whereas for the other dairy products the first dilution was enriched at 35 °C for 24 h and one loopful of the enriched culture was streaked onto BPA and incubated aerobically at 35 °C for 24–48 h. The swabs were incubated in TSB with 6.5% w/v NaCl for 18 h at 35 °C for enrichment; then a loopful was streaked onto BPA (detection limit <1 log CFU/g) and the plates were incubated at 35 °C for 24–48 h.

Four well-isolated, typical *S. aureus* colonies (black with and without opaque halo) were selected from each plate and transferred onto Tryptone Soya Yeast Extract agar (TSYEA; LAB M) for purification and further characterization. Preliminary identification was based upon Gram-staining, catalase reaction, mannitol fermentation, coagulase test, morphological and cultural characteristics. Each *S. aureus* strain was stored at −80 °C in TSB containing 20% glycerol. A sample (raw milk, swab or dairy product) was defined as positive if it contained at least one *S. aureus* isolate.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility towards 14 antimicrobials was determined by the agar-dilution method in Mueller-Hinton agar (Merck, Darmstadt, Germany) according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2009) and the results were evaluated after incubation at 35 °C for 24 h. The final plate concentrations (μg/mL) used were: beta-lactam antibiotics [penicillin, P (0.125); oxacillin, Ox (2); amoxicillin/clavulanic acid, Amx (0.5/0.25)], tetracyclines [tetracycline, T (2)], macrolides [erythromycin, E (2)], glycopeptides [vancomycin, V (2)], phenicols [chloramphenicol, C (8)], fluoroquinolones [ciprofloxacin, Cp (1)], folate pathway inhibitors [trimethoprim/sulfamethoxazole, Sxt (4/76); trimethoprim, Tm (4)], aminoglycosides [gentamycin, G (1); amikacin, Ak (16); kanamycin, K (8)], ansamycins [rifampicin, R (0.5)], according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC breakpoints (EUCAST, 2017). Multidrug-resistance was defined as previously proposed (Magiorakos et al., 2012). *S. aureus* ATTC 25923, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as control strains.

2.4. Molecular confirmation and genetic characterization of MRSA

2.4.1. Detection of the coa and nuc genes

All phenotypically confirmed MRSA strains were submitted to further molecular characterization. Extraction of genomic DNA from bacterial cultures was conducted according to the protocol of DNA purification from Gram-positive bacteria by the Pure Link Genomic DNA kit (Invitrogen, Carlsbad CA). *S. aureus* strains were confirmed by PCR targeting the *coa* (coagulase) and *nuc* (nuclease) genes (*S. aureus* species-specific determinants). A 500- to 650-bp fragment of the *coa* gene and a 416-bp fragment of the *nuc* gene were amplified using previously described primer sets (Hookey et al., 1998; Sudagıdan and Aydın, 2009) and PCR conditions (Zdragas et al., 2015). The PCR amplicons were separated in 1.5% agarose gels stained with ethidium bromide and visualized under UV illumination (TEX-20 M, Life Technologies, Gibco BRL System).

2.4.2. Detection of the mecA, mecC (mecA$_{LGA251}$) and Pandon-Valentine Leucocidin (PVL) genes

The detection of the *mec*A gene was achieved by PCR using the primers 5′-AAAATCGATGGTAAAGGTTGGC-3′, corresponding to nucleotides 1282 to 1303, and 5′-AGTTCTGCAGTACCGGATTTGC-3′, corresponding to nucleotides 1793 to 1814 (Murakami et al., 1991).

The PCR method described by Stegger et al. (2012) was performed for the detection of the *mec*C and PVL genes. The primers 5′-GAAAAAAAGGCTTAGAACGCCTC-3′ and 5′-GAAGATCTTTTCCGTTTTCAGC-3′ were used for the detection of the

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